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Cold Stress and Acute Organophosphorus Exposure: Interaction Effects on Juvenile Northern Bobwhite[1]

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Abstract. The interaction of cold stress and acute chlorpyrifos (*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate) exposure was examined in juvenile northern bobwhite (*Colinus virginianus* L.) as it relates to brain cholinesterase (ChE) activity and mortality. Temperatures spanned a 35.0°C optimum to a 27.5°C minimum; oral doses of chlorpyrifos ranged from 0.25 to 3.0 mg (8.3 to 100.0 mg/kg). Dose, temperature and body weight were significant factors in the ChE response. In addition, low temperatures potentiated dose-related ChE depression. Cholinesterase activity in non-surviving quail was inhibited at low temperatures but was not affected significantly by dose; dose, however, was the single factor which significantly influenced mortality. The $LD50_{4hrs}$ was approximately 91.3 mg/kg. These data demonstrate that cold stress augments certain toxic effects in juvenile northern bobwhite and should be considered when appraising pesticide hazards to wildlife.

Field applied pesticides occasionally result in substantial non-target animal kills despite prior testing which allegedly establishes their safety to wildlife. Toxic evaluations frequently center on chronic LC50 and acute LD50 tests (Gilbertson 1975; USEPA 1982). These procedures allow for appraisal of chemical effects under idealized laboratory conditions without regard for interactive environmental influences. Herein may lie a significant flaw in our ability to predict field response adequately.

Organophosphorus insecticides represent a class of acutely toxic biodegradable pesticides whose use has risen since the enforcement of restrictions on persistent organochlorine chemicals. One effect of organophosphorus poisoning is to inhibit cholinesterase (ChE) activity in exposed individuals. Acetylcholine, the enzyme substrate, is involved in thermoregulation (Meeter and Wolthius 1968); therefore, ChE inhibitors may affect temperature regulation and contribute to greater toxicity in animals exposed to temperature stress. In endothermic species, these impacts may be most pronounced in juveniles, since their homeostatic mechanisms are not yet fully developed and they have narrower thermoneutral zones than adults (Barott and Pringle 1946).

This study evaluates the interactive effects of cold temperatures and acute chemical insult by the organophosphorus insecticide chlorpyrifos (*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate) in juvenile northern bobwhite (*Colinus virginianus* L.).

Materials and Methods

Test Protocol

Four temperatures, chosen to evaluate cold stress in juvenile bobwhite (Table 1), bracket the approximate optimal brooding temperature (35.0°C) and the estimated minimal non-lethal ambient temperature (27.5°C) for 14-day old bobwhite as deter-

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Table 1. Average percent brain cholinesterase inhibition (relative to controls) in northern bobwhite, categorized by temperature and chlorpyrifos dose

Chlorpyrifos dose (mg/bird)	Temperature °C			
	35.0	32.5	30.0	27.5
Control	0	0	0	0
0.25	—[a]	—	0	—
0.50	8	5	3	2
0.75	—	—	—	13
1.00	16	11	19	18
1.25	—	—	—	31
1.50	28(1)[b]	31	43	50(1)
2.00	29(1)	39(1)	29(1)	76(1)
2.25	—	42(2)	—	—
2.50	25(2)	25(5)	43(5)	—
3.00	43(5)	—	—	—

[a] Dashes represent non-tested doses within temperature
[b] Numbers in parentheses represent fatalities based on a sample size of 10 individuals per cell

mined in preliminary studies. They also represent temperatures potentially experienced by bobwhite under natural field conditions (Rosene 1969).

Six concentrations of chlorpyrifos and a corn oil control solution were administered by gavage to groups of 10 birds per dose per test temperature. The quantity of active ingredient received per bird ranged from 0.25 to 3.0 mg (8.3 to 100.0 mg/kg) and varied within groups maintained at each temperature based upon hypothesized maximum non-lethal levels (Table 1).

Experiments were performed in a 3.07 × 2.74 × 2.39 m Forma Scientific environmental chamber (Model #74833A)[3] programmed for 10 air exchanges per hour. Lighting was provided by cool white fluorescent overhead lights set on a 12L:12D cycle. Bobwhite were housed in 76 × 61 × 25 cm galvanized steel cages constructed of 1.27 × 2.54 cm steel mesh with solid steel side panels. Fecal pans were suspended below each cage and in combination with the side panels prevented visual contact among cages. Deionized water and Purina Game Bird Startena® were provided *ad libitum*, and water was changed daily. Birds at each dose level were housed in separate cages for a total of 7 cages per temperature.

Four days prior to insecticide administration, 10-day old bobwhite were identified with monel #1 wing tags, weighed, and separated, regardless of sex, into 10 progressive weight categories. One bird from each category was assigned randomly to each cage and conditioned in the environmental chamber at 35.0°C.

When bobwhite reached 14 days of age, test temperatures were initiated at the onset of the light cycle. Birds were weighed and given 0.3 ml of pure corn oil or chlorpyrifos/corn oil solution. Dosage volume was based on 1% of the approximate mean bird weight (30 g) at dosing. Four hr after gavage, birds were asphyxiated by CO_2, re-weighed, and sexed. Preliminary studies revealed that 4 hr post-gavage approximates the time of maximum chlorpyrifos induced ChE inhibition in juvenile northern bobwhite. Most organophosphorus induced fatalities also may occur within this interval (Grue and Shipley 1984).

[3] Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the USEPA.

Chlorpyrifos Preparation

Chemical doses were prepared from technical grade Dursban® (94% chlorpyrifos) and pure corn oil diluent. Chlorpyrifos was mixed in corn oil so that the desired dose was contained in 0.3 ml of solution. Prepared solutions were heated at 55°C for 2 hr and shaken overnight on a Burrell® wrist-action shaker.

To evaluate the accuracy of the test solution preparation, duplicate samples of each dose were diluted in hexane and analyzed on a Hewlett-Packard® 5830 gas chromatograph equipped with a glass column packed with 10% OV210 on Chromasorb® W HP 80/100 mesh. Test solutions analyzed to within 9% of target concentration.

ChE Assays

Upon sacrifice, bobwhite brains were removed, wrapped in aluminum foil, and frozen in liquid nitrogen. Maximum time between sacrifice and ChE analysis was 23 days ($\bar{x}$ = 8 days).

Cholinesterase activity was determined colorimetrically by the method of Ellman *et al.* (1961), using acetylthiocholine iodide (Sigma Chemical Co., St. Louis, MO) as the substrate. A macro VirTis® model 45 homogenizer with upright blades was used to mascerate Trizma buffered brain tissue (Busby *et al.* 1983). Homogenate centrifugation was performed on a Sorvall® RC-5 superspeed refrigerated centrifuge at 5°C for 20 min at 20,000 g. Cholinesterase activity was determined on a Perkin-Elmer® Lambda 1 UV/VIS spectrophotometer set at 412 nm; enzyme activity was expressed in µmol of substrate hydrolyzed/min/g wet weight brain. The mean value of duplicate assays was calculated for each sample. Duplicate PRECILIP® ChE standards (Boehringer Mannheim Diagnostics, Indianapolis, IN) were made for each temperature series analysis.

Statistical Analyses

Cholinesterase response for all bobwhite was analyzed by the regression model

$$R = b_0 + b_1W + b_2D + b_3D^2 + \sum_{i=1}^{3} b_{i+3}I_i + \sum_{i=1}^{3} b_{i+6}I_iD + \sum_{i=1}^{3} b_{i+9}I_iD^2 \quad (1)$$

where R = predicted µmoles acetylthiocholine iodide hydrolyzed/min/g wet weight brain
W = final bird weight (g)
D = dose (mg)
I_1 = {1 if temperature = 32.5°C; 0 if otherwise}
I_2 = {1 if temperature = 30.0°C; 0 if otherwise}
I_3 = {1 if temperature = 27.5°C; 0 if otherwise}

Parameter estimates (b_i) were obtained by standard least squares procedures and were tested for significant difference from zero by the t-test (Draper and Smith 1981). Predictor variables with

Table 2. Significant parameter estimates in the final regression model (see Equation 1 in text) for northern bobwhite brain cholinesterase activity

Parameter[a]	Variable	Parameter estimates	S.E. of parameter estimates	Simple r^{2b}
b_0	Constant	3.607	—	—
b_1	Weight	0.085	0.022	0.047
b_2	Dose	−1.137	0.153	0.523
b_4	32.5°C	1.132	0.344	0.003
b_5	30.0°C	1.328	0.322	0.011
b_6	27.5°C	2.083	0.343	0.008
b_{10}	32.5°C × D^2	−0.276	0.091	0.046
b_{11}	30.0°C × D^2	−0.277	0.093	0.051
b_{12}	27.5°C × D^2	−0.916	0.145	0.076

[a] All p-values < 0.01
[b] Overall r^2 = 0.63

parameter estimates not significantly different from zero (α = 0.05) were dropped stepwise from the full model. Differences between parameter estimates for individual temperatures also were assessed by t-test. The described analysis was conducted a second time for the non-surviving subset of the original bobwhite group. A variation on the above regression was explored initially for possible sex or sex by dose interaction effects on ChE activity.

The proportion of dead individuals resulting from the influences of dose and temperature were analyzed by binomial regression with the PROBIT link function (McCullagh and Nelder 1983)

$$\Phi^{-1}(p) = a_0 + a_1D + a_2T + a_3D \cdot T \qquad (2)$$

where $\Phi^{-1}(\ )$ = inverse of the normal cumulative probability function
p = proportion of dead individuals
D = mg of chlorpyrifos/bird
T = temperature (°C)

Approximate tests of significance follow McCullagh and Nelder (1983).

More detailed experimental methods and quality assurance procedures are filed in the Quality Assurance Office, USEPA Environmental Research Laboratory, Corvallis, Oregon.

Results

Final body weight, insecticide dose, and ambient temperature significantly affected bobwhite ChE response with dose emerging as the single most powerful predictor (Table 2). Cholinesterase response to dose did not differ between sexes ($P > 0.5$).

Comparisons among the individual temperature equations derived from the initial regression model indicate that the 35.0°C regression line differed from all remaining temperatures by the absence of a significant quadratic term (Figure 1). In addition, the regression curves for 30.0 and 32.5°C were not significantly different ($P > 0.5$), and the 27.5°C curve was significantly lower than all other temperature curves ($P < 0.01$). Thus, as temperature decreased, increasing doses of chlorpyrifos caused progressively greater suppression of brain ChE activity (Figure 1).

Maximum average ChE inhibition, 76%, occurred in birds which ingested 2.0 mg of chlorpyrifos at 27.5°C, resulting in only one mortality (Table 1). However, dosage groups with the greatest mortality exhibited only 43% ChE inhibition. Twenty percent depression is routinely accepted as indication that exposure to a ChE depressant has occurred, while 50% depression is the prescribed level to reliably implicate a ChE depressant as the cause of death (Ludke *et al.* 1975). The data demonstrate great variability within these limits. Using the final regression model (Figure 1), the predicted chlorpyrifos doses which would prompt 20% ChE depression in juvenile bobwhite over the temperature range 35.0 to 27.5°C are 1.08 to 0.86 mg (36.0 to 28.7 mg/kg). Fifty percent depression over the same temperature range is predicted to require ingestion of 2.70 to 1.59 mg (90.0 to 53.0 mg/kg) chlorpyrifos.

Neither temperature nor the temperature by dose interaction were significant predictors of bobwhite mortality ($P = 0.40$ and $P = 0.28$, respectively). Thus, the final PROBIT link function for pooled temperatures was

$$\Phi^{-1}(p) = -3.972 + 1.417D \qquad (3)$$

where D = mg of chlorpyrifos/bird.

Approximate standard errors were 0.595 and 0.266 for the first and second parameter estimates, respectively. With temperatures pooled, bobwhite mortality first occurred with ingestion of 1.5 mg (50.0 mg/kg) chlorpyrifos (Table 1), and the estimated $LD50_{4hrs}$ was 2.8 mg/bird (91.3 mg/kg).

Of the variables monitored, only temperature significantly impacted ChE activity in non-surviving bobwhite ($P < 0.001$); greater ChE inhibition occurred at 27.5, 30.0 and 32.5°C ($P < 0.05$) than at 35.0°C (Figure 2). Cholinesterase depression was not significantly different among non-survivors at 27.5, 30.0 and 32.5°C, and averaged 87% inhibition.

Discussion

Cold stress potentiated dose-related ChE depression in juvenile northern bobwhite without increasing mortality in the 4 hr after exposure to chlorpyrifos. In addition, a standard level of ChE depression was not characteristic of cold stressed

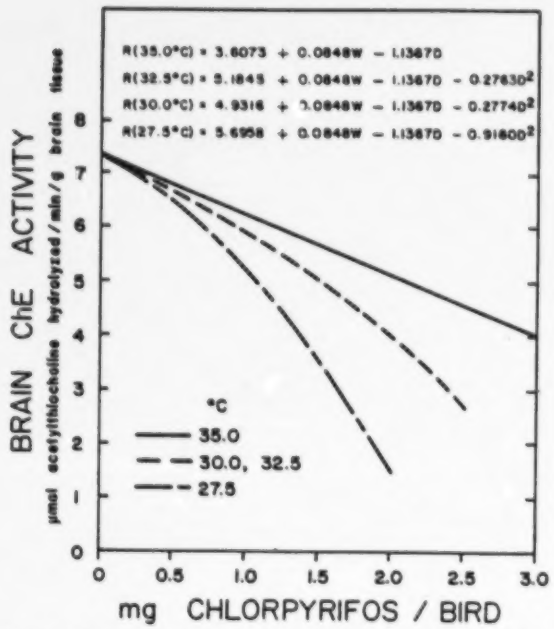

Fig. 1. Final regression equations for brain cholinesterase (ChE) activity (R) in juvenile northern bobwhite as a function of temperature (T) and chlorpyrifos dose (D). The 35.0°C regression line lacked a significant quadratic term. Plots of the regressions were standardized to a common control value (0 dose) since there were no significant differences in ChE activity of controls among temperatures; the mean regression coefficient for the 4 test temperatures was used. Weight (W) was standardized to 30 g for comparison. A single regression line is presented for 30.0 and 32.5°C as their regression equations did not differ significantly

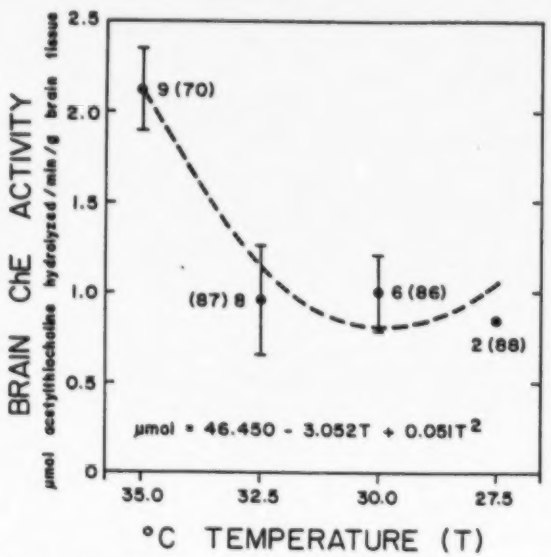

Fig. 2. Average brain cholinesterase (ChE) activity of non-surviving chlorpyrifos dosed northern bobwhite for test temperature, bracketed by one standard error. (No S.E. is available for the 27.5°C average, since the two ChE values were identical.) The number adjacent to each mean represents the number of individuals included in the average; the number within parentheses indicates the percent ChE depression based upon control (0 dose) levels. The best fit equation also is presented

and unstressed dead bobwhite, suggesting that decreased ChE activity was not the sole factor responsible for mortality.

Organophosphorus insecticides alter the tolerance of organisms to cold stress (Baetjer and Smith 1956; Marton *et al.* 1962; Rattner *et al.* 1982, 1987), possibly by impairing the thermoregulatory system. Lowered cold tolerance appears linked to hindered endocrine activity, particularly suppressed adrenocortical operation (Screbocan *et al.* 1971; Hart *et al.* 1973; Holmes *et al.* 1979; Rattner *et al.* 1982), while direct thermoregulatory impairment is associated with disrupted hypothalamic function (Coudray-Lucas *et al.* 1983). There is some indication that the primary effect is on regulatory processes related to raising body temperature (Meeter and Wolthius 1968; Ahdaya *et al.* 1976). Therefore, organophosphorus compounds pose the threat of hypothermia, because they intensify body temperature decline that accompanies cold exposure (Meeter 1971; Ahdaya *et al.* 1976; Rattner and Franson 1984).

If detoxification mechanisms are retarded during hypothermia, the availability of toxicant in the system should be relatively greater, resulting in greater ChE depression. In fact, cold stress did intensify ChE inhibition; yet, cold stress did not aggravate the short term ability of juvenile bobwhite to survive acute exposure to chlorpyrifos under the dose levels and time frame employed. Not all organophosphorus insecticides produce similar trends in survival and/or ChE activity when coupled with cold stress (Rattner *et al.* 1982, 1987; Fleming *et al.* 1985). Differences may be attributed to varying modes of action or metabolism, response time, and/or the range of temperatures explored as it relates to temperature endurance of the test subjects.

The trend toward greater ChE depression at lower temperatures was consistent between surviving and non-surviving bobwhite, although ChE levels were significantly lower in non-survivors (70 to 88% average depression). This suggests that severe ChE inhibition occurs rapidly and immediately prior to death, possibly the result of irreversible failure of the detoxification system. In addition, ChE depression in non-surviving bobwhite was not associated with dose, which implies that individuals have variable susceptibilities to toxic insult.

In conclusion, the salient impact of temperature on chlorpyrifos treated birds was an aggravated decrease in ChE activity. Mortality under the short duration of the test was largely unaffected by temperature although it was significantly influenced by dose. Therefore, if death is considered the primary toxic effects endpoint for wildlife species, temperature might be perceived to generate little effect on chlorpyrifos treated bobwhite. It is becoming increasingly more evident, however, that sublethal effects allude to potential long-term deleterious consequences. In the case of reduced ChE activity, evidence is mounting that behavioral activities can be modified, possibly resulting in reduced efficacy of survival mechanisms and/or reproductive strategies. Although it is largely recognized that acute hazard evaluation without dose-response considerations is a critical omission in toxicity assessments for wildlife (Hill and Camardese 1984), it is apparent that lack of regard for environmental influences also constitutes a notable deficiency.

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